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DNA barcoding supports the presence of the cryptic ocellated eagle ray, *Aetobatus ocellatus* (Myliobatidae), in French Polynesia, South Pacific

by

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Résumé. – Mise en évidence de la présence d'*Aetobatus ocellatus* (Myliobatidae) en Polynésie française grâce au barcode ADN.

L'espèce du genre *Aetobatus* présente en Polynésie française a été identifiée comme étant *A. ocellatus*, la raie aigle léopard. Ce résultat est basé sur le barcode ADN et valide ainsi la récente séparation de *A. narinari*, la raie aigle, en deux espèces distinctes. L'échantillonnage des raies a été effectué sur les archipels des Tuamotu et de la Société en 2012. Tous les échantillons ont le même haplotype, suggérant que ce sont des conspécifiques et qu'ils sont proches génétiquement des spécimens d'Australie et de Nouvelle-Calédonie.

Key words. – Myliobatidae - *Aetobatus narinari* - Myliobatidae - Cryptic species - DNA barcoding - CO1.

The spotted eagle ray *Aetobatus narinari* (Euphrasen, 1790) is a bentho-pelagic Myliobatidae species and was considered until recently to have a circumtropical distribution. Strong evidence based on genetic analyses suggested that the spotted eagle ray represented a species-complex with an Indo-Pacific lineage that was clearly distinct from the Central Atlantic and the Eastern Pacific lineages (Richards *et al*., 2009; Schluessel *et al*., 2010a; Cerutti-Pereyra *et al*., 2012). As a consequence, White *et al*. (2010), based on morphological and molecular data, split the species complex into two distinct cryptic species by resurrecting the ocellated eagle ray, *A. ocellatus* (Kuhl, 1823), as a valid species occurring in the Indo-West Pacific. This left *A. narinari* restricted to the Atlantic and Eastern-Pacific.

This recent taxonomic revision, with the division of *A. narinari* into two cryptic species and the distinction of populations with uncertainty concerning their range of distribution, can have further implication for fisheries management and conservation (Dudgeon *et al*., 2012). *A. narinari* is listed as "Near Threatened" by the IUCN Red List (Kyne *et al*., 2006), whereas the status of *A. ocellatus* has not yet been assessed. It is also not clear where, in the Pacific Ocean, the range of the two species separates or overlaps. Indeed, while samples have been compared between the Eastern Pacific and the Western Pacific, samples from the Central Pacific can clarify the geographic separation between the two species. The 6400 km oceanic expansion of the Pacific Ocean generally represents a genetic barrier between West and East Pacific for most neritic fish species (Schultz *et al*., 2008). Therefore, we hypothesize that the specimens from French Polynesia (Southern Central Pacific) likely belong to *A. ocellatus*.

The present study aimed to identify the *Aetobatus* species present in French Polynesia using DNA barcoding. Our study contributes to a better understanding of the distribution of species in the *Aetobatus* complex, which is necessary for a correct evaluation of the conservation status of the different species.

MATERIALS AND METHODS

Tissue samples were collected from the atolls of Rangiroa and Manihi in the Tuamotu archipelagos and from the islands of Moorea and Maupiti in the Society archipelagos (Fig. 1). Details regarding collection data and photos of sampled individuals are available in the Barcode of Life Data System (BOLD Systems, http://www.boldsystems.org). A piece of tissue was removed by cutting a small fin clip (about 1 cm^2) from captured individuals or using a modified spear gun with a biopsy tip mounted at the extremity allowing removal of a piece of tissue from the body of free-ranging individuals, a technique that has been successfully used on stingrays and sharks (Mourier *et al*., 2013). Tissue samples were preserved in 98% ethanol at 4°C and are now registered with field numbers (TCB) and collection numbers at the Station de biologie marine de Concarneau of the Muséum national d'Histoire naturelle (BPS) (Tab. I).

Global DNA was extracted following the modified method with CTAB (Jones, 1953). Homologous fragments of 652 bp encoding the cytochrome *c* oxidase subunit 1 (CO1) were amplified via a Polymerase Chain Reaction using the TripleMaster® System (Eppendorf) following the manufacturer's instructions. Fragments were obtained with primer pairs of PCR S156/R249 and S156/R84 (Iglésias and Lévy-Hartmann, 2012). We used the following thermic cycle parameters: pre-denaturation at 94°C for 2 min, 45 cycles of denaturation at 94°C for 1 min, hybridization at 54°C for 1 min,

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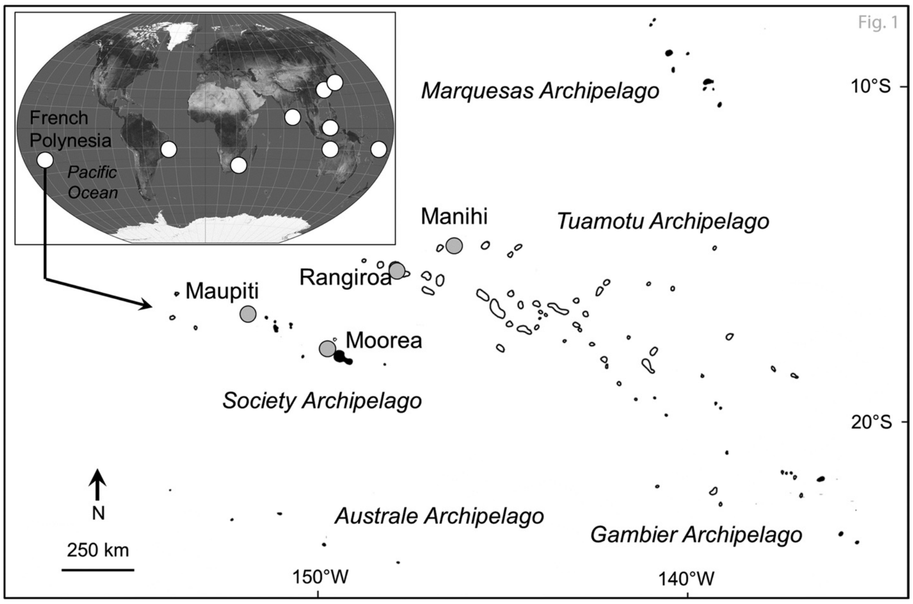

Figure 1. - Locations for sampled *Aetobatus ocellatus* (grey circles) in French Polynesia and for comparative materials (circles in insert: Australia, New Caledonia, Indonesia, India, South Africa, Brazil, Japan, Korea).

Table I. - References of samples and COI sequences used for the genetic analysis. * Primarily recorded in BOLD Systems as *Aetobatus* cf. *narinari*.

Locality	Tissue/DNA sample No.	GenBank Access. No.	BOLD Process ID	References
Aetobatus ocellatus				
French Polynesia, Manihi	TCB-001; BPS-2704	KT208286	AETO001-14	Present study
French Polynesia, Rangiroa	TCB-002; BPS-2705	KT208287	AETO002-14	Present study
French Polynesia, Moorea	TCB-004; BPS-2707	KT208288	AETO003-14	Present study
French Polynesia, Moorea	TCB-005; BPS-2708	KT208289	AETO004-14	Present study
French Polynesia, Maupiti	TCB-006; BPS-2709	KT208290	AETO005-14	Present study
French Polynesia, Maupiti	TCB-008; BPS-2711	KT208291	AETO006-14	Present study
Australia, Collier Bay	BW-A3015	EU398507	FOAF143-07	Ward *et al*., 2008
Indonesia, Lombok	BW-A2609	EU398508	FOAE397-06	Ward *et al*., 2008
India, Rameswaram	RMS-01	–	INELA022-12	Pavan-kumar *et al*., unpubl.
New Caledonia, Saint Vincent Bay	BPS-0656	–	ANCC001-13	Iglésias *et al*., unpubl.
Unknown locality	AU_41	JN184054	ANGBF2029-12	Aschliman *et al*., 2012
South Africa, Umhlanga	Smith 28.1 #3	JF492798	TZMSC556-06*	Steinke *et al*., unpubl.
Aetobatus narinari				
Brazil, São Paulo	LBPV53065	JQ365215	MFSP1964-11	Ribeiro *et al*., 2012
Brazil, São Paulo	LBPV53066	JQ365212	MFSP1965-11	Ribeiro *et al*., 2012
Brazil, São Paulo	LBPV53067	JQ365214	MFSP1966-11	Ribeiro *et al*., 2012
Brazil, São Paulo	LBPV53068	JQ365213	MFSP1967-11	Ribeiro *et al*., 2012
Aetobatus narutobiei				
Unknown locality	-	NC_022837	CYTC7664-15	Yang *et al*., unpubl.
Japan	-	AB291068	GBGC3822-07	Sasaki and Hamaguchi, unpubl.
Korea	FAfla050620-1	EU339362	GBGC6418-09	Yoon *et al*., 2009

and extension at 72°C for 3 min. At the end of electrophoresis, gel bands containing the PCR products were removed and purified using the QIAquick® gel extraction kit. Sequence reactions were realized using the BigDye® Terminator v3.1 (Applied Biosystems) sequence kit with different primers. The thermocycles were: predenaturation at 96°C for 2 min, 40 cycles of denaturation at 96°C for 0.5 min, hybridization at 50°C for 0.5 min, and extension at 60°C for 4 min. PCR and sequence reactions were conducted with a thermocycler TGradient (Biometra). The sequencing was realized with the genetic analyser ABI PRISMTM 310 (Applied Biosystems).

Connection of sequences, correction of electropherograms, and alignment of sequences were performed using Sequencher v4.2 (Gene Codes Corporation). Sequences contained no gaps or missing data. The final dataset involved 19 nucleotide sequences from the species *A. ocellatus*, *A. narinari* and *A. narutobiei* (White *et*

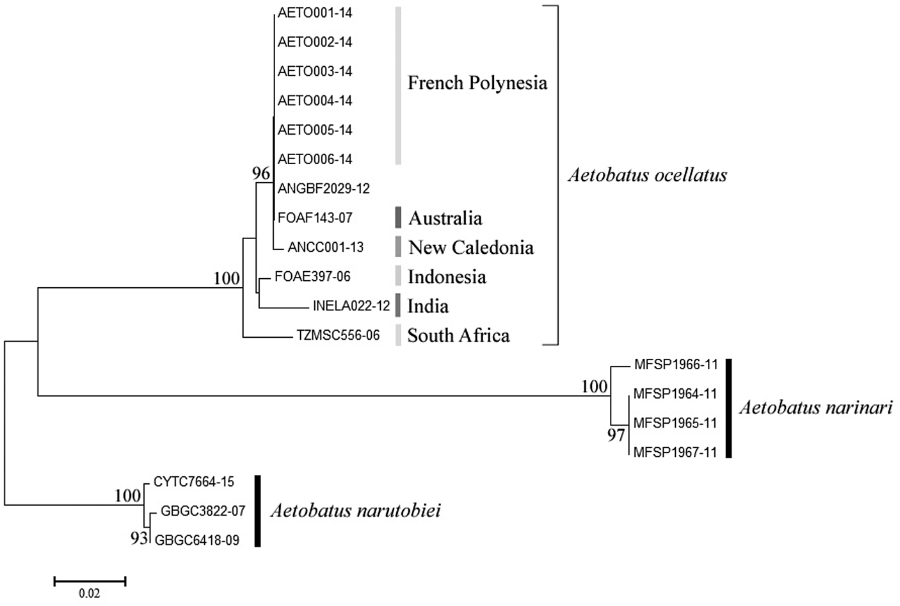

Figure 2. - Neighbour-Joining distance tree (K2p model) of the partial COI sequences (652 bp, 'barcode region') revealing the placement of individuals of *Aetobatus ocellatus* from French Polynesia within the *Aetobatus* complex. Specimens are labelled with their BOLD Process ID. Bootstrap values over 75% are indicated above branches.

al., 2013). The COI sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank) and in the BOLD Systems under Accession No. and BOLD Process ID, respectively listed in table I. Sequences in the BOLD Systems are part of the project '*Aetobatus ocellatus* identification' (AETO). Evolutionary analyses were conducted in MEGA v5.05 (Tamura *et al.*, 2011). The sequences from our specimens were aligned with available sequences from other *Aetobatus* specimens to construct a Neighbour-Joining (NJ) tree (Saitou and Nei, 1987) using a distance matrix of Kimura 2-parameter (Kimura, 1980) of genetic sequence similarity. Codon positions included were 1^{st}+2^{nd}+3^{rd}. Bootstrap proportions for the NJ tree were determined with 10,000 pseudoreplicates to estimate each node's robustness. Sequences of the species *A. narutobiei* (according to the recent revision of White *et al.*, 2013) were used as an outgroup to root the tree. The evolutionary model of Kimura 2-parameter (K2p) was also used to estimate the evolutionary divergence between sequences and the average evolutionary divergence over sequence pairs within and between groups.

RESULTS

All DNA barcodes of eagle rays from samples obtained from the various locations in French Polynesia have a similar haplotype, suggesting they are all conspecifics. These individuals merge into a cluster of species identified as *A. ocellatus* (Fig. 2). DNA barcoding provides strong support for species discrimination of *A. ocellatus* and *A. narinari* concordant with previous results (Richards *et al.*, 2009; White *et al.*, 2010). The intraspecific average evolutionary divergence was 0.9% for *A. ocellatus* and 0.3% for *A. narinari*, and the interspecific average evolutionary divergence was 23.7% for *A. ocellatus*/*A. narinari*. Individuals from French Polynesia share a strong genetic similarity with individuals from Australia and New Caledonia (Fig. 2). The highest intraspecific distance occurred between the individuals from French Polynesia and an individual from South Africa, corresponding to the most distant analysed locations. The intraspecific divergence observed within *A. ocellatus* is about 78 times lower than the interspecific divergence observed between *A. ocellatus* and *A. narinari*, clearly matching the tenfold rule proposed by Hebert *et al.* (2004) and supporting *A. ocellatus* as a distinct species.

DISCUSSION

The present study supports the occurrence of the ocellated eagle ray, *A. ocellatus*, in French Polynesia (Society and Tuamotu archipelagos). *Aetobatus ocellatus* is considered by the IUCN Red List (under the uncorrected name *A. narinari*) as "Vulnerable" in South-East Asia, and the species complex is considered "Near Threatened" globally (Kyne *et al.*, 2006). The status of both *A. ocellatus* and *A. narinari* should therefore be reassessed, specifically because each species will be exposed to different threats in their currently limited geographic range. Slow growth, late sexual maturity, and slow reproduction (over 12 months of gestation producing only a few pups) (Schluessel *et al.*, 2010b) make *Aetobatus* species highly vulnerable to human fishing and other pressures. In French Polynesia, *A. ocellatus* is involved with two kinds of human activities. First, they are important for tourism activities, being commonly targeted for scuba diving and snorkelling activities, mostly in channels of atolls and islands. At the same time, Planes *et al.* (2006) showed that eagle rays are one of the predators of the Black-lipped pearl oyster *Pinctada magaritifera* (Linnaeus, 1758). This oyster represents the second economic pillar of French Polynesia through pearl farming. Better knowledge of the ocellated eagle ray could help determine new ways to protect the local economy while preserving the species.

Our results do not rule out the possibility that *A. narinari* could occur in Polynesian waters. A similar situation recently occurred within the genus *Manta* with both *Manta alfredi* (Krefft, 1868) and *M. birostris* (Walbaum, 1792) being present in the Marquesas Islands (Mourier, 2012), while *M. alfredi* is the dominant *Manta* in the other archipelagos despite anecdotal sightings of *M. birostris*. Indeed, more samples need to be collected throughout the three other Polynesian archipelagos to complete the dataset initiated by the present study. However, results from this study have shown that the samples we collected belong to the same species and are genetically close to individuals from Australia and New Caledonia. Comparisons with samples from other areas such as Indonesia, India, or South Africa suggest that a particular genetic signature may distinguish populations. Increasing the number of samples and locations over a larger area would certainly increase our understanding of the population structure of both *A. ocellatus* and *A. narinari*.

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